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Roles of Calcium Ions in Hyphal Tip Growth

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INTRODUCTION

Fungal hyphae extend by tip growth, a process also common in numerous specialized plant cells (reviewed in reference 57). Fungi that grow as fission or budding yeasts also demonstrate transient tip growth during cell elongation or bud formation (58) and therefore will be considered in this review. Tip growth is characterized by a dynamic equilibrium between the synthesis and expansion of cell wall and plasmalemma and the application of expansive force derived from turgor pressure or the cytoskeleton. Regulation of this equilibrium is extraordinarily precise and localized because it generates a constant-diameter tube and occurs only in the most apical 5 μm of a growing tip. This extreme polarization of hyphae applies not only to their growth pattern but also to their morphology, organelle positioning, and cytoskeletal and ion distributions. Therefore, tip growth involves localized cell wall deposition (5, 44), cell wall "setting" (166), the bulk forward migration of cytoplasm (73, 98, 99, 123), maintenance of the polarized distribution of organelles (43, 45), and regulation of the properties of the actin cytoskeleton (2, 71). Ca^{2+} ions have been proposed to regulate and coordinate many of these processes. They are also involved in other aspects of fungal physiology and differentiation (119) which may or may not directly relate to tip growth. These aspects will not be discussed here.

Hyphal tips contain polarized distributions of both free (37) and membrane-associated (98, 141, 171) Ca^{2+} ions, which have received a lot of attention because of their regulatory potential. Similarly, Ca^{2+} gradients are also often associated with cell polarity in other highly polarized cells (142) such as zygotes and rhizoids of algae, pollen tubes and root hairs of higher plants (13, 24, 76, 87, 102, 109, 121, 124–127, 165) and neurites of animal cells (26). All of these tip-growing cells have a higher concentration of Ca^{2+} in their apices than in their subapical regions. From arguments initially devised by Jaffe et al. (77–79) and further developed by Picton and Steer (116, 117, 146), the tip-high gradient of Ca^{2+} distribution is thought to play one or more roles in establishing and maintaining apical organization, morphogenesis, and growth. In this review we examine the evidence for the polarized distribution of Ca^{2+} in growing hyphae, consider possible mechanisms for the generation and maintenance of this gradient, and suggest functions for these ions in hyphal growth.

CALCIUM DISTRIBUTION

In this section we shall review what is known about the calcium distribution in growing hyphae and critically discuss the techniques that have been used to detect calcium.

Cytoplasmic Free Ca^{2+}

In fungal hyphae it is the distribution of free calcium that is of greatest interest, since this is the form which plays a central role in signaling and regulation in other systems (19) and thus has the potential to coordinate apical growth. Imaging of free Ca^{2+} was revolutionized by the development of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)-based fluorescent Ca^{2+} indicators which have high affinity and selectivity for free Ca^{2+} (158). The distribution of cytoplasmic free Ca^{2+} can be determined by using two types of fluorescent dyes. The "ratio" dyes shift either their excitation (Quin-2, Fura-2) or their emission

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(Indo-1) spectra upon binding Ca^{2+}. By measuring the ratio between the intensity of fluorescence at the Ca^{2+}-bound and Ca^{2+}-free wavelengths, it is possible to determine the concentration of free Ca^{2+} independently of possible variations in dye concentration in different parts of a cell. Furthermore, these dyes can be calibrated to measure the free Ca^{2+} concentration (47). In contrast, single-wavelength dyes, such as Fluo-3, change only fluorescence intensity, not wavelengths, when bound to Ca^{2+}. The fluorescence intensity of these dyes depends on both dye and Ca^{2+} concentrations; therefore, regions of cells with a larger accumulation of dye (154) will fluoresce more intensely, regardless of the Ca^{2+} concentration. The ratio dyes are thus more suitable for detecting a Ca^{2+} gradient.

The use of Ca^{2+}-imaging dyes in fungal hyphae has proven to be difficult. Attempts to load these dyes by a variety of techniques, such as electroporation of *Saprolegnia* hyphae (75a), microinjection of *Uromyces* germlings (122), and loading of the cell-permeant forms of these indicators into *Saprolegnia* hyphae (75a) and *Neurospora* hyphae (141), have all either failed to load the dye or resulted in dye-containing but nongrowing hyphae. However, recently Read et al. (122) succeeded in loading dye into *Peziza* and *Sordaria* hyphae by microinjection. The hyphae continued to grow with apparently normal morphology but at unspecified rates. We have also loaded Fluo-3 and Indo-1 into *Saprolegnia* hyphae by using the acid-loading technique of Bush and Jones (15). Dye-loaded hyphae resumed growth, and half had normal morphology. However, growth rates were lower than normal, especially with Indo-1, branching frequency was often higher, and half the tips were abnormally swollen (75a). In *Saprolegnia* hyphae, the staining pattern appears nonpunctate (see Fig. 2b), as expected if the dye is located in the cytoplasm. However, in both *Peziza* and *Sordaria* hyphae, some dye is apparently cytoplasmic but much more is punctate, suggesting sequestration in unidentified organelles, even though dextran-conjugated dye, developed to avoid the problem (102), was used (122). In both the *Saprolegnia* and *Peziza-Sordaria* work, growth with normal morphology indicates the usefulness of the dye-loaded hyphae but the abnormalities in *Saprolegnia* hyphae illustrate both the need for caution in the use of the dyes and the importance of Ca^{2+} in tip growth. In contrast to the difficulties encountered in dye loading of hyphae, yeast cells have been successfully loaded with Indo-1 and Fura-2 by using acid loading (50) and electroporation (68), respectively. Indo-1 loaded to the cytoplasm, whereas Fura-2 appeared to be in both the cytoplasm and vacuoles. However, in neither of these studies was there sufficient resolution or analysis to contribute to the question of bud-specific gradients of Ca^{2+}. For further details on the loading and use of these Ca^{2+} dyes, see references 18, 122, and 159.

Bearing in mind the above problems, it has nevertheless been possible to show a tip-high gradient of cytoplasmic free Ca^{2+} in actively growing *Saprolegnia* hyphae stained with Indo-1 (37). The Ca^{2+} concentration was highest at the very tip (within about 3 μm of the tip) and declined rapidly to a lower level 10 to 20 μm behind the tip (Fig. 1). This gradient correlated with growth, being absent in nongrowing hyphae (37). The shape of the gradient depended on the external Ca^{2+} concentration. At 10^{-3} (37) or 10^{-5} M exogenous Ca^{2+}, there was a high level of Ca^{2+} in the extreme tip, which produced a very steep gradient (Fig. 1A), but the tip level was much lower at 10^{-6} M exogenous Ca^{2+} (Fig. 1B), thus producing a much shallower gradient. A comparably steep gradient may exist in *Peziza* hyphae (122), but absence

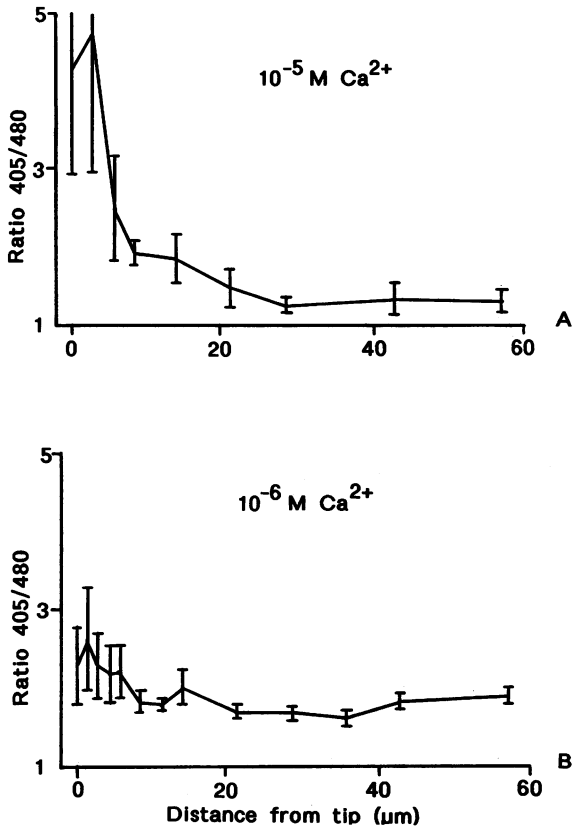

FIG. 1. Distribution of cytoplasmic free Ca^{2+} in growing hyphae of *Saprolegnia ferax* stained with Indo-1. Hyphae were loaded with Indo-1 as previously described (37). The ratio of the fluorescence intensity at 405 and 480 nm was determined along a transect through the middle of each hypha. This ratio is proportional to the cytoplasmic free Ca^{2+} concentration. Hyphae were mounted and observed in either OM growth medium, which contains 10^{-5} M Ca^{2+} (98) (A) or a defined medium containing 10^{-6} M Ca^{2+} (37) (B). All hyphae were growing with average growth rates of 5.25 ± 2.13 μm/min (n = 4) (panel A) and 3.03 ± 0.86 μm/min (n = 6) (panel B). Note that the exogenous medium influences the steepness of the cytoplasmic free Ca^{2+} gradient near the tip of the hyphae. Values are means ± 1 standard deviation (69a).

of ratio data and information on exogenous Ca^{2+} levels in the preliminary report preclude a detailed comparison with data obtained with *Saprolegnia* hyphae.

In contrast to the gradient observed for Indo-1, growing *Saprolegnia* hyphae containing the single-wavelength dye Fluo-3 showed fluorescence to be lower in the tip, not reaching a maximum until 3.3 ± 1.3 μm behind the apex (Fig. 2a). It seems likely that this pattern reflects the distribution of Fluo-3. The tapered shape of the tip and the large number of vesicles concentrated in the apex (59) both result in less cytoplasm (as opposed to membrane-enclosed organelles and vesicles) and thus less dye. This could lead to weak apical fluorescence, even in the presence of a high concentration of free Ca^{2+}; this indicates the limitation of a single-wavelength dye. A further limitation with Fluo-3 is seen in the sensitivity of the dye-loaded hyphae to the exciting light. Hyphae often ceased growing and showed an increase in fluorescence intensity, which was most pronounced 3 to 9 μm behind the tip (Fig. 2c), in the mitochondrion-rich region of the hypha (172). The fluorescence in-

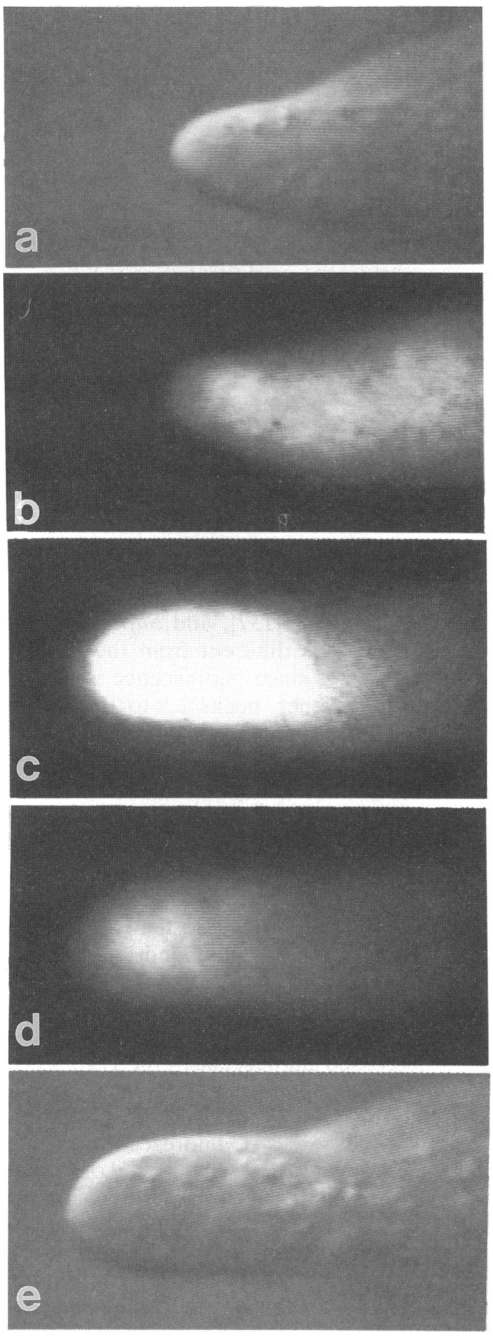

FIG. 2. Growing hypha of *S. ferax* loaded with the single-wavelength Ca^{2+}-imaging dye Fluo-3 (69a). (a) Nomarski image of a hyphal tip growing at 2.1 μm/min. (b) Fluorescence image of panel a, showing low fluorescence in the extreme tip. (c) The same hypha 3 min after panel b was obtained. Note that the same exposure was used and that the intensity of fluorescence increased, especially in the region 3 to 9 μm behind the tip. (d) Shorter exposure of panel c to show the location of the region of most intense fluorescence. This region peaked at a mean of 3.3 μm behind the tip in 15 hyphae examined. (e) Normarski image 256 s after panel a. Magnification, ×1,440.

crease in this zone may indicate light-induced release of Ca^{2+} from mitochondria (see the section on Ca^{2+} homeostasis in growing hyphae, below), but, at present this is speculative. Callaham and Hepler (18) also noted cellular damage in irradiated dye-loaded cells, thus reinforcing the need to consider such problems and keep the time of observation and intensity of excitation light to a minimum.

Steep, tip-high cytoplasmic free Ca^{2+} gradients, similar to those described above in hyphae, have been found in growing pollen tubes. The gradient was highest and steepest in the apex and declined 10 to 20 μm (102), 22 to 65 μm (121), or 50 to 100 μm (109) behind the tip. These observations differ from earlier findings in pollen tubes, algal rhizoids, and root hairs, in which the gradient was either absent or very shallow over a region of 300 μm or more (12, 13, 24, 64, 108, 129). The manner of data collection in the latter studies may not have resolved a steep gradient at the extreme tip. Alternatively, since the steep tip-high gradient of free Ca^{2+} is coincident with pollen tube and hyphal extension (37, 102, 109, 121), cessation of growth may explain the absence of a Ca^{2+} gradient in these reports because the cells were not shown to be growing when observed. Reports of the actual Ca^{2+} concentration at the tip versus the subapical regions range from 100 (tip) versus 20 nM (subapical) (108) to 1,700 to 2,600 (tip) versus 100 nM (subapical) (109). Ca^{2+} microelectrodes measured the cytoplasmic free Ca^{2+} concentration to be 2,500 nM in the tip versus 300 nM in subapical regions (13). However, accurate calibration of fluorescent Ca^{2+} indicators and electrodes is difficult; therefore, it is better to think of these Ca^{2+} values as relative and not absolute. Since the concentration of cytoplasmic free Ca^{2+} in plant cells is similar to that in fungi (see the section on cellular Ca^{2+} homeostasis, below), it seems likely that the cytoplasmic free Ca^{2+} gradient in fungi will be within the range established for tip-growing plant cells.

Spatial free Ca^{2+} gradients such as those observed in tip-growing cells could be simulated by indicator-associated artifacts (157). Ca^{2+} gradients can be mimicked by compartmentalization of dyes into organelles (8, 16, 157), inaccurate photometry and improper use of imaging systems (8, 157), and cytoplasmic microenvironments which hinder dye binding (157). The first three problems are readily detectable or avoidable (157). However, differences in microenvironment are not always expected or apparent, and the techniques suggested to identify them (i.e., use of ionophores [157]) are also likely to alter the microenvironment, thus making it difficult to detect. It is likely that microenvironments of both pH and cytoplasmic consistency exist in a growing hypha. Low pH decreases the dissociation constant of Ca^{2+} indicators, which can result in little change in dye fluorescence in the presence of a large increase in the intracellular Ca^{2+} concentration (90). Reported pH gradients in fungi indicate that the tip may be acidic (97, 161); therefore, the cytoplasmic free Ca^{2+} concentration in the *Saprolegnia* tip may be higher than indicated by the Indo-1 ratio (37). Therefore, the cytoplasmic pH should also be monitored to ensure accuracy in determining the cytoplasmic free Ca^{2+} concentration. Cytoplasmic viscosity also affects the binding constant of Ca^{2+} dyes, with an increase in viscosity leading to a lower fluorescence ratio in the absence of a change in Ca^{2+} concentration (133). There is a possible localized region of elevated cytoplasmic viscosity stretching 8 to 30 μm behind the tip of growing *Saprolegnia* hyphae, as suggested by the high concentration of F-actin in this region (72, 74). However, the Ca^{2+} gradient observed in *Saprolegnia* hyphae (37)

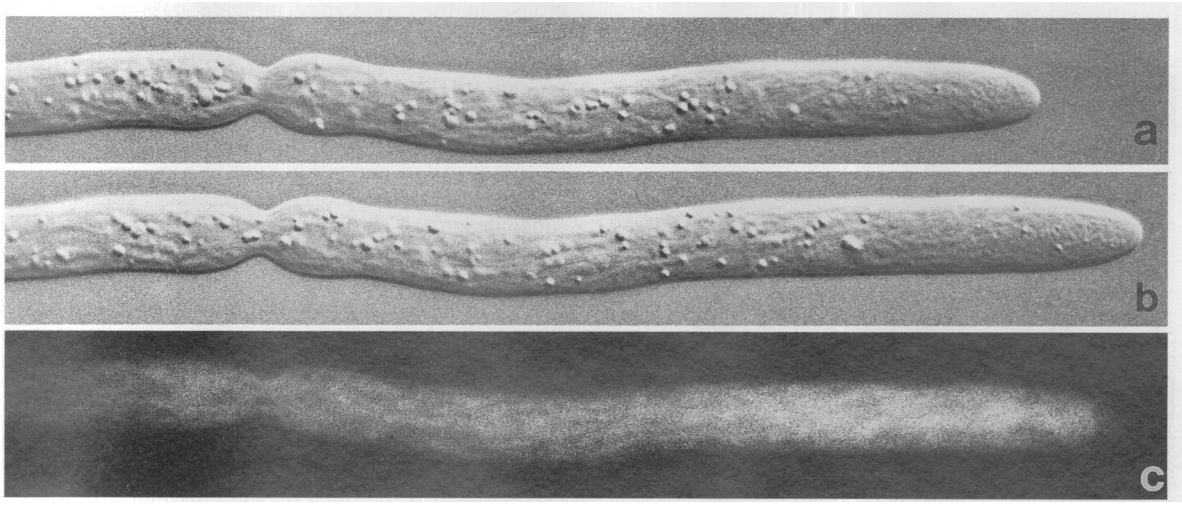

FIG. 3. Growing hypha of *S. ferax* loaded with CTC and photographed at 0 (a) and 1 min (b) (172a). (c) Membrane-associated Ca^{2+}-CTC distribution in the same hypha seen in panel b (172a). Fluorescence is low in the tip and peaks approximately 8 μm behind the apex. The hypha had been stained with CTC for 3 min, and its growth rate was 12.5 μm/s. Magnification, ×1,121.

does not change with viscosity (i.e., it is not high on either end of the putative viscous zone).

Membrane-Associated Ca^{2+}

Chlortetracycline (CTC) has been used extensively to visualize Ca^{2+} distributions in cells (20), including hyphae. However, because it only fluoresces when bound to Ca^{2+} in the vicinity of a membrane, it is not a useful indicator of free cytoplasmic Ca^{2+}. Nevertheless, it is a valuable indicator of membrane-associated Ca^{2+} which may indicate which organelles are involved in Ca^{2+} homeostasis. CTC is well suited to the visualization of Ca^{2+} accumulated in vesicles and organelles (152), since the fluorescence intensity depends on the concentrations of CTC, Ca^{2+}, and membrane (33). Since Ca^{2+}-CTC is membrane impermeable, unlike free CTC, at equilibrium Ca^{2+}-CTC accumulates in organelles which contain higher concentrations of free Ca^{2+} (33). For these reasons Ca^{2+}-CTC membrane-associated fluorescence primarily indicates the presence of calcium-sequestering organelles and/or a higher density of membrane. Ca^{2+}-CTC membrane-associated fluorescence is further complicated by pH. pH affects the ability of CTC to both cross membranes (enhanced by acidic pH) and bind Ca^{2+} (enhanced by basic pH) (152). An organelle with an acidic interior would thus tend to show poor Ca^{2+}-CTC fluorescence, since it would accumulate less CTC and the Ca^{2+}-CTC present would not fluoresce as brightly. It is therefore important to consider variations in pH when interpreting CTC data, just as it is for observations on free cytoplasmic Ca^{2+}.

Although CTC binds Mg^{2+} as well as Ca^{2+}, the fluorescence in cells is thought to visualize primarily Ca^{2+}. The increase in fluorescence is much greater on binding of Ca^{2+} than of Mg^{2+} in the vicinity of a membrane (51), and CTC binds Ca^{2+} more readily than it binds Mg^{2+} (21). However, since the concentration of Mg^{2+} is often high in cells, it is advisable to confirm that the observed fluorescence distribution is due to Ca^{2+}, either by altering Ca^{2+} concentrations (70, 87, 171) or by using filters which can monitor the influence of Mg^{2+}- versus Ca^{2+}-induced fluorescence (139).

Fungal hyphae stained with CTC show membrane-associated Ca^{2+} to be highest near the tip (*Achlya* [125], *Basidiobolus* [98], *Neurospora* [137], and *Saprolegnia* [70, 171] hyphae). However, it is different from the distribution of cytoplasmic free Ca^{2+}, since fluorescence is not highest in the extreme tip but, rather, peaks 2.5 to 10 μm behind the apex (98, 125, 137), a point first made clear by Yuan and Heath (171) (Fig. 3). The absence of this pattern of fluorescence in earlier work (70) may be due to the washing and staining treatment just before observation, which could have stopped growth and thereby altered the Ca^{2+} distribution. Careful comparison of Ca^{2+}-CTC with membrane-associated fluorescence in growing *Saprolegnia* hyphae allowed Yuan and Heath (171) to conclude that peak fluorescence was associated mainly with mitochondria and possibly the endoplasmic reticulum. When the exogenous Ca^{2+} concentration was high, Ca^{2+}-CTC membrane-associated fluorescence increased, whereas a low exogenous Ca^{2+} concentration caused decreased fluorescence (70, 171). This implies that Ca^{2+} ions accumulated in mitochondria during hyphal growth and that these Ca^{2+} ions were releasable.

The observation of low CTC fluorescence in the very tip (Fig. 3), a region known to have a high level of free Ca^{2+} ions (see the section on cytoplasmic free Ca^{2+} ions, above) and to be occupied by large numbers of wall vesicles (59), was at first surprising. Neither Ca^{2+} concentration nor membrane abundance should limit fluorescence. There are several possible explanations for this low apical fluorescence. Since the tip is tightly packed with wall vesicles, fluorescence may be low as a result of a small accessible volume of cytoplasm limiting the concentration of CTC (154). The tip may be acidic (53, 85, 97, 161), which would depress Ca^{2+}-CTC membrane-associated fluorescence. A third possibility is that the concentration of Ca^{2+} sequestered in mitochondria is much higher than the free Ca^{2+} concentration in the growing tip. Since cytoplasmic Ca^{2+} ions are presumed to be in the nanomolar range (see the sections on cytoplasmic free Ca^{2+} ions and Ca^{2+} homeostasis in growing hyphae) and sequestered Ca^{2+} may reach millimolar levels (33), this seems a likely explanation for the relatively low fluorescence in the tip.

Ca^{2+}-CTC membrane-associated fluorescence has been observed in other types of tip-growing cells. Some cells show fluorescence to be highest in the very tip (moss cauloneama [125, 139], pollen tubes [126], algal germlings [87], and filamentous algae [112]). Others, like fungal hyphae, show greater fluorescence a few micrometers behind the tip (root hairs [125] and pollen tubes [128]). This disparity between cell types may be the result of varying cytoplasmic pH (128) or may be due to differing states of growth as discussed above for observations of free cytoplasmic Ca^{2+} concentration.

Ultrastructural Distribution of Calcium

Ultrastructural knowledge of the calcium distribution may indicate which organelles are actively involved in sequestering Ca^{2+} or functioning as Ca^{2+} sinks during hyphal growth. There are a variety of analytical electron microscopy techniques which can be used to detect calcium. X-ray microanalysis (XRMA) is now the most widely used of these techniques. The limit of resolution for XRMA is about 50 nm, and the sensitivity is such that it is probably only capable of detecting sequestered calcium (115). XRMA has been used to detect calcium in polyphosphate granules in chemically fixed and sectioned fungal specimens (106). However, Ca^{2+} ions are small and highly soluble; therefore, during fixation, embedding, and sectioning, most free Ca^{2+} ions are washed out (167) or redistributed (113). Loss of ions can be minimized using freeze-substitution with a nonpolar substitution fluid such as ether (65). However, observation of frozen hydrated specimens is preferable, since it avoids fixation-induced loss and rearrangement of ions. XRMA of frozen hydrated material has been used to detect ions (other than Ca^{2+}) in fungi (66). Thus there is the potential to detect unperturbed Ca^{2+} ions.

Electron energy loss spectroscopy has a spatial resolution of 0.5 nm and is also about 2 orders of magnitude more sensitive than XRMA (6, 115, 143). Although this technique is better suited to detecting low concentrations of ions and localizing them within the cell, it may not prove to be the best technique for detecting Ca^{2+}. It requires ultrathin (30- to 50-nm) sections, which means that frozen hydrated material cannot be used. Furthermore, as pointed out by Hodson and Sangster (65), even if material is prepared by freezing followed by anhydrous substitution and embedding, soluble ions are likely to be lost during sectioning since ultrathin sections must be cut onto water.

Particle-induced (or proton-induced) X-ray emission analysis also has greater analytical sensitivity than XRMA, and it can be used with frozen hydrated material (65). Unfortunately, resolution is low (1 μm), and therefore it is not useful for localizing calcium to specific organelles. However, it can provide information about the overall distribution of calcium. For example, it was used to demonstrate that total cellular calcium was concentrated in the tips of pollen tubes (124, 127). However, chemical fixation was likely to have distorted this distribution.

Pyroantimonate precipitation of Ca^{2+} has also been used to localize these ions. In *Phycomyces* sporangiophores, calcium pyroantimonate precipitates were observed in a number of organelles, including mitochondria, endoplasmic reticulum, vacuoles, and Golgi body equivalents (107). However, fixation-induced redistribution of ions was likely to have occurred prior to precipitation, and the technique lacks the sensitivity to give information on free cytoplasmic Ca^{2+} concentration.

As can be seen in this section, there are many pitfalls in the techniques for identifying the distributions and concentrations of Ca^{2+} in growing hyphal tips. For this reason, there are rather few compelling reports and numerous uncertainties. However, it is fair to conclude that for both hyphae and other tip-growing cells, there is reasonable evidence for a tip-high gradient of free cytoplasmic Ca^{2+}, whose absolute concentration is apparently influenced by exogenous Ca^{2+} levels. This gradient is correlated with tip growth, being dissipated in nongrowing tips.

Ca^{2+} HOMEOSTASIS IN GROWING HYPHAE

How is the tip-high steady-state Ca^{2+} gradient established and maintained during hyphal growth? To begin to answer this question, we shall briefly review Ca^{2+} homeostasis in animal and plant cells in general and follow this up with what is known about Ca^{2+} homeostasis in fungi. We shall then describe a model for Ca^{2+} homeostasis in fungi, which is tailored to explain the tip-high gradients of cytoplasmic free Ca^{2+} and membrane-associated Ca^{2+}.

Cellular Ca^{2+} Homeostasis

Generally, Ca^{2+} ions enter the cytoplasm passively (moving down their concentration gradient) either through Ca^{2+} channels in the plasma membrane or through Ca^{2+} channels in the membranes of Ca^{2+}-sequestering organelles such as the endoplasmic reticulum, mitochondria, and vacuoles (36, 149). Ca^{2+} ions are cytotoxic at high levels and must therefore be tightly controlled in cells (19). Typical cytoplasmic free Ca^{2+} concentrations are 100 to 200 nM for animal cells (19), 100 to 300 nM for plant cells (40, 80), and 100 nM (68, 101) to 350 nM (50) for fungi. It should be pointed out that these are average values and do not take into account the higher values reported in growing tips (range, 100 to 2,600 nM; see the section on cytoplasmic free Ca^{2+} ions, above). Nevertheless, the maintenance of low cytosolic Ca^{2+} concentration is widespread. Cytosolic Ca^{2+} ions are maintained at low levels, either by pumping Ca^{2+} out of the cell through the plasma membrane or by sequestering them into organelles. Sequestered calcium may either remain as a releasable Ca^{2+} pool or become part of a nonreleasable calcium sink. Although a great deal is known of the characteristics and locations of Ca^{2+} channels and pumps of animal cells (3), less is known about plants (80) and even less is known about fungi (101).

Mechanisms for Ca^{2+} Entry into Fungal Cells

Ca^{2+} entry into the cytoplasm is thought to occur primarily at the plasma membrane, where stretch-activated channels permeable to Ca^{2+} (as well as other ions) have been identified and characterized by patch clamping in *Saccharomyces* (49), *Uromyces* (173), and *Saprolegnia* (38) cells. Garrill et al. (37, 38) devised a novel method of preparing protoplasts which permitted the study of channels in protoplasts derived from different regions of the hypha. Stretch-activated channels permeable to Ca^{2+} were found at a higher density in tip-derived protoplasts (representing the first 90 μm from the tip) than in those originating from more posterior regions. This asymmetric distribution of stretch-activated Ca^{2+} channels may bias Ca^{2+} entry into the hypha. A similar nonuniform distribution of channels may also occur in *Saccharomyces* cells, because Gustin et al. (49) reported that some membrane patches lacked channels, but

their technique precluded identification of the cellular origin (bud versus mother cell) of these patches. Other types of Ca^{2+} channels may also be present in the plasma membrane and play a role in Ca^{2+} entry as is implied by inhibitor experiments (see the section on variations in influx of exogenous Ca^{2+}, below). However, as far as we know, such channels have not been identified in electrophysiological investigations of fungal cell membranes.

Maintenance of Low Cytosolic Ca^{2+} Levels in Fungi

An effective way of keeping the cytosolic Ca^{2+} concentration low is to remove it from the cell through the plasma membrane. It is not yet clear whether fungi do this by using primary transport systems, such as a Ca^{2+}-ATPase (134) or an ATP-driven H^+/Ca^{2+} exchanger (101) or via secondary transport systems, such as an H^+/Ca^{2+} antiport involving a H^+ gradient (39, 150). However, we are not aware of evidence demonstrating that fungi excrete Ca^{2+}; therefore, the function of Ca^{2+} export has not been established either for tip growth or for maintenance of the Ca^{2+} gradient. Ca^{2+} ions may also be maintained at low levels in the cytoplasm by sequestration into organelles. The vacuole of fungal cells is thought to be an important site for Ca^{2+} storage because it contains a high concentration of Ca^{2+} (28, 50). Ca^{2+} ions accumulate in plant vacuoles via a H^+/Ca^{2+} antiport driven by a proton-ATPase (9), and they are also likely to do so in fungi (35, 110). In growing fungal hyphae the vacuole has the potential to act as an infinitely expandable Ca^{2+} sink. As the hypha extends, the vacuole continually enlarges, and therefore its capacity to store Ca^{2+} continually increases.

Mitochondria can also accumulate Ca^{2+}. However, mitochondria isolated from animal cells only do so when exposed to pathologically high Ca^{2+} concentrations, i.e., 700 μM (81). Therefore, mitochondria are not considered to sequester Ca^{2+} in healthy animal cells. Likewise, Moore and Akerman (105) and Pitt and Ugalde (119) conclude that mitochondria are probably not significant in Ca^{2+} sequestration in either plant or fungal cells. However, Kauss (83) views the issue as still open for the situation in plant cells. The highest reported free Ca^{2+} in a tip-growing cell is 28 μM (13), which is well below the putative activation threshold for mitochondrial accumulation of Ca^{2+}. Furthermore, *Saccharomyces* petite mutants with defective mitochondria maintain Ca^{2+} balance similar to that in wild-type cells (35). Thus, biochemical evidence implies that mitochondria do not sequester Ca^{2+} during tip growth. In contrast, the membrane-associated Ca^{2+} distribution of intact normally growing hyphae (shown by CTC fluorescence) and the presence of calcium pyroantimonate deposits in mitochondria (107) imply that mitochondria do accumulate Ca^{2+} as described in the section on membrane-associated Ca^{2+}. Similarly, an extending hyphal tip contains a dividing population of mitochondria, some of which are left behind to populate subapical regions of the cell as the tip grows forward. In *Saprolegnia* hyphae there is the equivalent of one mitochondrion per micrometer of mature hypha, with each mitochondrion having an approximate volume of 2 μm^3 (58a). Assuming that mitochondria can accumulate 100 mM Ca^{2+} (a consensus measurement of this value is not available; literature values range from 0.02 [22] to 1,272 mM [104] for plant and animal mitochondria, calculated from cited values per milligram of protein by using a value of 1 mg of mitochondrial protein equals 1 μl [137] for isolated mitochondria), for each micrometer of hyphal extension, mitochondria are capable of sequestering 1.2×10^8 Ca^{2+} ions. The relevance of this number is best seen relative to possible Ca^{2+} influx rates via the stretch-activated Ca^{2+} channels referred to above. With a mean channel flux value of 12.47×10^6 ions per s (38) and a channel density of 8/μm^2 (37), and assuming that the mean channel open time is 6% (derived from Fig. 5 in reference 38), that the growth rate is 10 μm/min, and that only channels in the apical dome are active in Ca^{2+} influx, one can calculate an influx rate of 56×10^8 ions per μm of growth. This calculation contains too many assumptions for high confidence but does show that the rate of Ca^{2+} sequestration by mitochondria may be significant relative to likely rates of Ca^{2+} influx. Therefore, the population of mitochondria could in theory contribute significantly to a Ca^{2+} sink. Picton and Steer (118) also provide evidence for Ca^{2+} sequestration by mitochondria in growing pollen tubes. There is thus a discrepancy between biochemical and cytological data as to the Ca^{2+}-sequestering function of mitochondria. Although CTC-membrane-associated fluorescence may change in response to pH, as opposed to Ca^{2+} (see the section on membrane-associated Ca^{2+}, above), it is also plausible that isolated mitochondria accumulate Ca^{2+} differently from those in an intact cell. Therefore the role of mitochondria in fungal Ca^{2+} homeostasis remains an open question.

The endoplasmic reticulum functions to sequester Ca^{2+} in animals (3) and may also do so in plants (36) via a high-affinity Ca^{2+}-ATPase which can maintain cytoplasmic Ca^{2+} at nanomolar levels. Thus the endoplasmic reticulum has the potential to maintain cytoplasmic Ca^{2+} at the observed low levels. Although the role of the endoplasmic reticulum in Ca^{2+} homeostasis of fungi has not been investigated, these organelles are likely to function similarly to those of animals and plants. This conclusion is supported by the pyroantimonate deposits found in the endoplasmic reticulum by Morales and Ruiz-Herrera (107).

Ca^{2+} sequestered by organelles may be releasable back to the cytoplasm. Vacuoles isolated from *Neurospora* cells release Ca^{2+} in response to inositol 1,4,5-trisphosphate (IP_3) exposure (27) and therefore may do so in intact hyphae. In contrast, animal cells possess a specialized endoplasmic reticulum that functions as an IP_3-releasable Ca^{2+} store (3). However, in the only fungus examined for this activity, isolated endoplasmic reticulum did not release Ca^{2+} in response to IP_3 (27). Fungi and plants appear to have similar mechanisms for Ca^{2+} homeostasis, since IP_3 induces the release of sequestered Ca^{2+} from plant vacuoles but not from the endoplasmic reticulum (80).

Another possible component of Ca^{2+} regulation is formed from the plethora of Ca^{2+}-binding proteins present in the cytoplasm (i.e., calmodulin; also see the section on Ca^{2+} regulation of the F-actin network, below). The numbers of these proteins, their location, and the extent to which they bind Ca^{2+} will also influence Ca^{2+} availability in the cytoplasm. Such Ca^{2+} buffering could regulate cytoplasmic Ca^{2+} in specific regions of the cytoplasm and thus generate Ca^{2+} gradients.

Establishment and Maintenance of the Ca^{2+} Gradient

A tip-high gradient could be initiated by either an unbalanced influx or efflux of Ca^{2+} (61, 84). Ca^{2+} influx is likely to occur at the tip as stretch-activated Ca^{2+}-permeable channels are present in greater numbers in the apical membrane (37, 38; see also the section on mechanisms of Ca^{2+} entry into fungal cells, above). This apical influx of Ca^{2+} appears to be important for the generation of the tip-high cytoplasmic

free Ca^{2+} gradient (see the section on cytoplasmic free Ca^{2+} ions, above), since blocking of these channels with Gd^{3+} results in the loss of the gradient (37). An apical influx of Ca^{2+} appears to be common to tip-growing organisms, as has recently been demonstrated for pollen tubes (88) and root hairs (140) by using a Ca^{2+}-specific vibrating electrode. Maintenance of the gradient presumably requires subapical removal of Ca^{2+} from the cytoplasm, either by pumping out through the plasma membrane or sequestration into organelles. Ca^{2+} pumps and exchangers in the plasma membrane are good candidates for this job, since they could permanently remove Ca^{2+} from the hypha. To maintain the gradient, such pumps and exchangers would not require biased distribution or activation providing that Ca^{2+} influx remained localized in the tip. Ion currents which are directed inward at the growing hyphal apex and outward in subapical regions (86) imply a role for ion pumps and exchangers in growth. However, this current is primarily involved with amino acid or nutrient transport (31, 53). Therefore, the role of Ca^{2+} pumps and exchangers in the maintenance of the Ca^{2+} gradient remains to be established.

Sequestration of Ca^{2+} by organelles localized just behind the tip, such as the endoplasmic reticulum (102) and mitochondria (145, 171), has also been hypothesized to play a role in generating the Ca^{2+} gradient. These organelles are expected to have the capacity to function as a Ca^{2+} sink (see the section on maintenance of low cytosolic Ca^{2+} levels in fungi, above), whose size increases as the hypha grows. Similarly, the vacuole also undoubtedly functions as a Ca^{2+} sink (see above), which also continues to enlarge during hyphal growth. The fact that the cytoplasmic free Ca^{2+} concentration declines only a few micrometers behind the apex but the vacuole is located far behind this region argues against a simple accumulation of Ca^{2+} directly into the vacuole in order to generate that Ca^{2+} gradient. However, vacuoles come from unidentified precursors, possibly the endoplasmic reticulum or special vesicles. If these precursors were in the cytoplasm ahead of the obvious vacuoles, they could take up Ca^{2+} in the region where the Ca^{2+} gradient begins to decline and deposit their sequestered Ca^{2+} into the vacuole upon fusion.

At present, it is not known how the Ca^{2+} ions, which probably show biased entry at the tip, are removed from the cytoplasm, thus sustaining the tip-high free Ca^{2+} gradient. However, a number of plausible mechanisms exist and are summarized in Fig. 4.

WHAT IS THE FUNCTION OF THE TIP-HIGH Ca^{2+} GRADIENT?

Typically, Ca^{2+} acts as a signal through a burst or waves of transiently increased cytosolic free Ca^{2+} concentration, which will initiate a cellular response. For example, elevated levels of IP_3 in guard cells of higher plants caused a transient increase in cytosolic free Ca^{2+} concentration, which, in turn, induced stomatal closure (42). Likewise, waves of increased Ca^{2+} concentration crossed the cytoplasm of eggs from the point of sperm entry, thus initiating egg activation (144). The gradient of cytoplasmic free Ca^{2+} concentration in tip-growing cells is different in that it apparently remains constant. The function of this gradient is unknown. A common approach to the investigation of the role of the Ca^{2+} gradient in fungal hyphae has been to observe the effects of disrupting this gradient by various means. A second approach has been the study of mutants which lack the typical Ca^{2+} gradient or have an abnormal Ca^{2+}-binding protein. In this section we discuss what has been learned from both of these approaches.

Variations in Influx of Exogenous Ca^{2+} Ions

Decreased Ca^{2+} influx. Experiments involving restriction of the influx of exogenous Ca^{2+} ions, either by chelating external Ca^{2+} ions or by blocking Ca^{2+} channels in the plasma membrane with agonists, are most easily interpreted as showing that movement of Ca^{2+} into the hypha is required for normal growth. When external Ca^{2+} ions were chelated with EGTA, *Neurospora* (137), *Fusarium* (131), and *Saprolegnia* (70) hyphae showed a marked reduction in extension rate and eventually ceased to extend. Furthermore, extension was abnormal, since the tips would intermittently swell, resulting in irregular hyphal morphogenesis (70, 131) (Table 1). Under these conditions the branching frequency of *Fusarium* hyphae was also increased (131). External Ca^{2+} ions influence the distribution of internal Ca^{2+} ions, since hyphae had diminished Ca^{2+}-CTC membrane-associated gradients (70, 171) and diminished free Ca^{2+} gradients (Fig. 1b) (see the section on cytoplasmic free Ca^{2+} ions) when exogenous Ca^{2+} concentrations were low.

Swollen hyphae induced by low exogenous Ca^{2+} coincided with both a loss of cell wall-bound Ca^{2+} (137) and a dramatic reduction and rearrangement of the apical F-actin network (70). Both the cell wall and the F-actin network are likely to be involved in apical morphogenesis. However, it is not clear whether external Ca^{2+} ions act directly on the wall or indirectly by altering the internal Ca^{2+} concentration, affecting the actin cytoskeleton and other growth processes. The site of Ca^{2+} action has been investigated by attempting to lower endogenous Ca^{2+} levels while leaving the exogenous Ca^{2+} concentration at normal levels. This can be achieved, at least in theory, by using Ca^{2+} channel blockers, which prevent Ca^{2+} from crossing the plasma membrane into the cytoplasm. Different types of Ca^{2+} channel blockers have been used, and many affect hyphal growth, morphogenesis, and branching in a manner similar to that for low exogenous Ca^{2+} levels (Table 1). This implies that the observed effects are due to altering the endogenous Ca^{2+} distribution and not the external wall-bound Ca^{2+}. It also seems likely that more than one type of Ca^{2+} channel is involved in growth, since drugs which block stretch-activated Ca^{2+}-passing channels (Gd^{3+} [37]), dihydroperidine-sensitive Ca^{2+} channels (nifedipine, nicardipine [132]), verapamil-sensitive channels (32), and channels sensitive to inorganic Ca^{2+} channel antagonists (e.g., cobalt [132]) all affect hyphal growth. However, most of these studies have not monitored intracellular Ca^{2+} levels, and Ca^{2+} channel blockers may not always have the predicted effect on cytosolic Ca^{2+}, possibly because of nonspecific effects (30). Recently, the Ca^{2+} channel blockers nifedipine, nicardipine, verapamil, bepridil, and Gd^{3+} were all shown to directly affect the activity of K^+-selective channels in plant plasma membranes (155). These were not secondary effects mediated by blockage of Ca^{2+} channels. Since these drugs blocked K^+ channels when used at concentrations less than 1 μM, the practice of using low concentrations of inhibitor cannot be taken as a guideline to ensure inhibitor specificity. This point is especially important since K^+ ions are likely to be involved in establishing cell polarity, at least in *Fucus* zygotes (67). Therefore, the response of cellular Ca^{2+} to the drug must be monitored together with morphological and growth responses. This has rarely been done, but there are two such studies. Stretch-activated Ca^{2+}-passing channels,

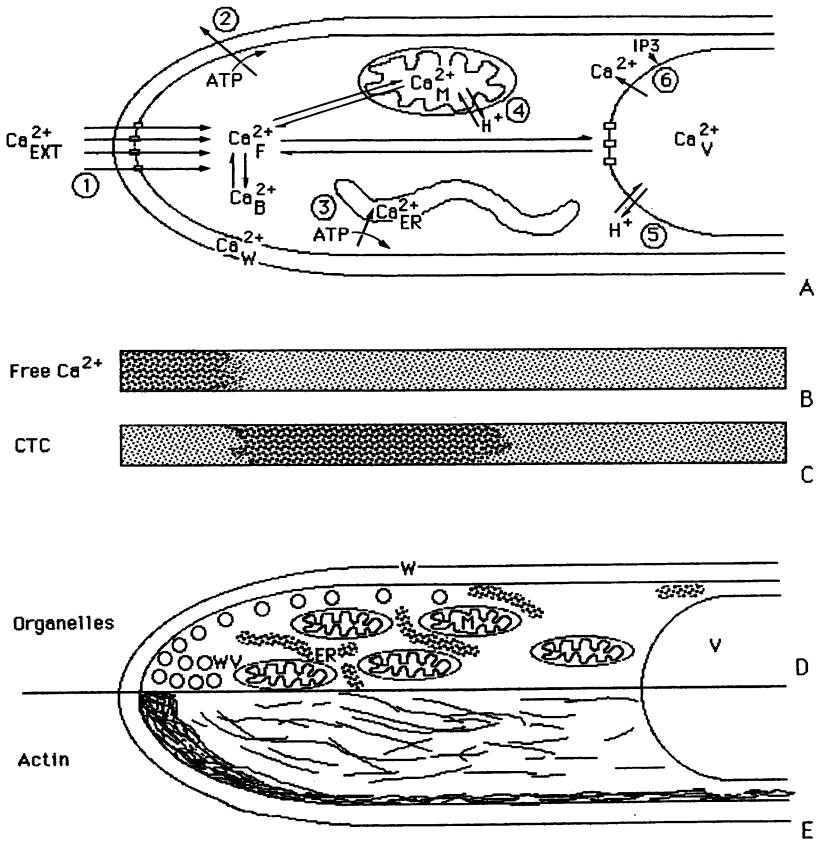

FIG. 4. (A) Potential factors involved in fungal Ca^{2+} homeostasis. Ca^{2+}_{Ext}, external Ca^{2+}; Ca^{2+}_{W}, Ca^{2+} bound to the cell wall; Ca^{2+}_{F}, cytosolic free Ca^{2+}; Ca^{2+}_{B}, Ca^{2+} bound to protein; Ca^{2+}_{ER}, Ca^{2+} sequestered in the endoplasmic reticulum; Ca^{2+}_{M}, Ca^{2+} sequestered in the mitochondria; Ca^{2+}_{V}, Ca^{2+} sequestered in the vacuole; 1, stretch-activated Ca^{2+}-passing channels; 2, Ca^{2+}-ATPase which pumps Ca^{2+} out of the cytosol (additional mechanisms for Ca^{2+} removal across the plasma membrane are discussed in the text); 3, Ca^{2+}-ATPase which sequesters Ca^{2+} into the endoplasmic reticulum; 4, H^+/Ca^{2+} exchanger which accumulates Ca^{2+} into the mitochondria; 5, H^+/Ca^{2+} antiport which accumulates Ca^{2+} into the vacuole; 6, IP_3-stimulated release of Ca^{2+} sequestered in the vacuole. Ca^{2+} enters the hypha at the tip, generating a localized region of high cytoplasmic free Ca^{2+} concentration. This Ca^{2+} is then either pumped out of the hypha, bound by protein, or sequestered into organelles to maintain the tip-high gradient of free Ca^{2+}. The cytoplasmic free Ca^{2+} concentration (B) and membrane-associated Ca^{2+} concentration (C) are compared with the model for Ca^{2+} homeostasis (A) and the distribution of organelles (D) and the F-actin network (E). Abbreviations: ER, endoplasmic reticulum; M, mitochondria; W, cell wall; WV, cell wall vesicles; V, vacuole.

but not K^+ channels, were blocked with Gd^{3+} in *Saprolegnia* protoplasts, as monitored by patch clamping (37). Gd^{3+} also rapidly dissipated the tip-high gradient of cytoplasmic free Ca^{2+} ions (monitored by using Indo-1) and caused hyphae to stop growing. Therefore, the cessation of hyphal extension could be associated with the loss of the intracellular Ca^{2+} gradient. In a second example, swelling and enhanced branching of *Neurospora* hyphae in response to verapamil was associated with dispersion of the Ca^{2+}-CTC membrane-associated gradient (32). Increasing the concentration of exogenous Ca^{2+} in the presence of verapamil enabled hyphae to resume their normal shape and branching, demonstrating that reduced influx of Ca^{2+} was most probably responsible for the swelling and the increased branching.

The effects of lowering the internal Ca^{2+} concentration by reducing apical influx should be mimicked by lowering the cytoplasmic free Ca^{2+} concentration with Ca^{2+} buffers. This has been found in *Saprolegnia* hyphae, in which loading with the EGTA-based dyes, Indo-1 and Fluo-3, decreased hyphal growth rates and induced hyphal swelling (see the section on cytoplasmic free Ca^+ ions, above). Similarly, in pollen tubes, microinjection of the Ca^{2+} buffer BAPTA [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] dissipated the cytoplasmic free Ca^{2+} gradient and blocked tip extension (102). Thus buffering of free Ca^{2+} does affect tip extension.

The role of Ca^{2+} influx in tip extension and branching is further supported by the morphology and branching of the frost and spray *Neurospora* mutants. These mutants are apparently deficient in Ca^{2+} uptake. They lack a Ca^{2+}-CTC membrane-associated gradient, grow slowly, display hyperbranching, and have abnormal swollen hyphae. The role of Ca^{2+} is apparent since high levels of exogenous Ca^{2+} allow mutants to assume normal growth rates and branching patterns (32). This further indicates that reduced influx of external Ca^{2+} ions was responsible for increased branching, slow growth, and abnormal hyphae.

Enhanced Ca^{2+} influx. The effects of enhancing the Ca^{2+} gradient or increasing cytoplasmic free Ca^{2+} may also give clues to the role of Ca^{2+} in tip growth. Increasing the external Ca^{2+} concentration generally results in an increased rate of hyphal extension and hyphae of normal apical morphology (70, 131, 137) and in a decreased frequency of branching (131) (Table 1). Very high concentrations of

TABLE 1. Summary of the effects of perturbing Ca^{2+} on hyphal growth[a]

Treatment	Expected effect	Monitored effect on internal Ca^{2+}	Tip extension rate[b]	Branching frequency[b]	Tip morphology[b]
Modulation of exogenous Ca^{2+}					
Low (0–10^{-7} M)	Decreased influx of exogenous Ca^{2+}	CTC fluorescence decreased [1, 2]	− [1, 3, 4, 5]	+ [5]	A [1, 3, 5]
Optimal (10^{-5}–10^{-2} M)	Influx of exogenous Ca^{2+}	"Normal" CTC fluorescence [1, 2]	N [1, 5, 6]	− [5]	N [1, 5]
High (>10^{-2} M)	High influx of exogenous Ca^{2+}	CTC fluorescence much increased [1, 2]	− [1]	ND	N [1]
Ca^{2+} channel antagonists					
Gd^{3+}	Block stretch-activated Ca^{2+} channels, reduced Ca^{2+} influx	Free Ca^{2+} decreased at extreme tip [7] or no effect on ^{45}Ca uptake or CTC fluorescence [3]	− [7, 9], N [3]	+ [9]	N [3, 8]
Verapamil	Block Ca^{2+} channels, reduced Ca^{2+} influx	Gradient of CTC fluorescence dissipated [10] or unchanged [3]	− [10], N [3]	+ [10]	A [10], N [3]
Others	Block Ca^{2+} channels, reduced Ca^{2+} influx	^{45}Ca uptake and CTC fluorescence unchanged [3]	− [9, 11], N [3]	+ [6, 11]	N [3]
Ca^{2+} ionophore (A23187)					
30–80 nM	Equilibrate endogenous Ca^{2+} with exogenous Ca^{2+}, which varied considerably, e.g. 36 [3], 700 [4], 250 [12], 0–1,000 [13], ? [11, 14] nM	ND[b]	+ [11]	− [11]	ND
>5 μM	Same	Dissipated CTC fluorescence gradient [3, 13], released ^{45}Ca[3] or ND	− [3, 11, 12, 13, 14]	+ [3, 4, 11, 12]	N [3, 14]
Inhibitors of calmodulin	Prevent Ca^{2+}-calmodulin-based regulation	Not applicable	− [11, 15]	+ [11, 15]	A [15], ND [11]
Inhibition of the phosphoinositide cycle	Block IP_3-induced release of Ca^{2+} from organelles, reduce endogenous Ca^{2+}	ND	− [16]	+ [16]	ND

[a] 1, *Saprolegnia ferax* (70); 2, *Saprolegnia ferax* (171); 3, *Neurospora crassa* (141); 4, *Neurospora crassa* (130); 5, *Fusarium graminearum* (27); 6, *Zoophthora radicans* (95); 7, *Saprolegnia ferax* (37); 8, *Saprolegnia ferax* (36a); 9, *Metarhizium anisopliae* (148); 10, *Neurospora crassa* (32); 11, *Fusarium graminearum* (132); 12, *Achlya bisexualis* (56); 13, *Saprolegnia ferax* (82); 14, *Phycomyces blakesleeanus* (135); 15, *Neurospora crassa* (114); 16, *Neurospora crassa* (52).

[b] Symbols and abbreviations: +, increased; −, decreased; N, normal; A, abnormal; ND, not determined; ?, exogenous Ca^{2+} levels unknown but probably higher than free cytoplasmic levels because they were not buffered.

external Ca^{2+} (>50 mM) will inhibit tip extension (70). It is not clear whether this inhibition is due to direct Ca^{2+} interactions with the cell wall (i.e., Ca^{2+}-induced rigidity of the apical cell wall [70]) or to a general toxic response to high cytosolic Ca^{2+} concentration. Higher concentrations of external Ca^{2+} lead to greater Ca^{2+}-CTC membrane-associated fluorescence (70, 171) and a steeper gradient of cytoplasmic free Ca^{2+} at the very tip (compare Fig. 1a and b) as well as bathing the wall in a higher Ca^{2+} concentration.

The cytosolic free Ca^{2+} concentration can, in theory, be raised by using the ionophore A23187, which is expected to equilibrate external and internal free Ca^{2+} levels. Addition of A23187 at concentrations above 5 μM has been shown to dissipate the Ca^{2+}-CTC membrane-associated gradient (98, 137, 171) and induce a net loss of $^{45}Ca^{2+}$ from hyphae (137). It appears, therefore, that the ionophore induces a loss of Ca^{2+} from organelles as well as equilibration across the plasmalemma. A23187 affects hyphal growth, influencing the extension rate, branching frequency, and cytoplasmic movement. When the cytosolic Ca^{2+} concentration was raised by using >5 μM A23187, hyphal extension was either partially or completely inhibited while branching was enhanced (Table 1). However, at lower concentrations of ionophore, the growth rate increased while branching was reduced (132), a response similar to that induced by elevated external Ca^{2+} levels. The complete disruption of the gradient with high concentrations of ionophore may inhibit elongation and induce branching, whereas lower concentrations may mimic the effects of elevated external Ca^{2+} concentrations, showing an increased growth rate and decreased branching frequency. These correlated effects on growth rates and branching frequencies may provide some indication of a possible role for the Ca^{2+} gradient in branch initiation, but at present the data are a composite from too many species examined under different conditions to make any speculation worthwhile. High intracellular Ca^{2+} concentrations can also induce cytoplasmic contractions. These contractions are always unidirectional toward the tip and can be induced by elevating cytoplasmic Ca^{2+} levels with A23187 or by UV microirradiation-mediated Ca^{2+} influx (73, 82, 98). We have suggested that these contractions may be an exaggerated form of the tipward cytoplasmic migration which normally occurs during growth.

When exogenous Ca^{2+} is absent, addition of A23187 is not expected to lead to an equilibrium elevation of the cytoplasmic free Ca^{2+} concentration. However, exposure to 25 μM A23187 in the absence of external Ca^{2+} also caused hyphae to cease extending and induced cytoplasmic contractions (82). This implies that a transient rise in the cytoplasmic Ca^{2+} concentration from organelles can induce predicted

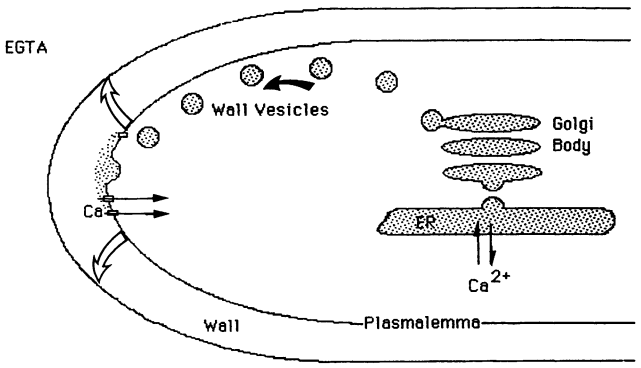

FIG. 5. Model describing how endogenous Ca^{2+} stores in the endoplasmic reticulum (ER) may be cycled into the Golgi apparatus and then to wall vesicles, which undergo directed transport to the apex. At the tip, wall vesicles fuse with the plasma membrane, thus depositing their contents into the periplasmic space. Ca^{2+} in these vesicles may provide a source of exogenous Ca^{2+} for Ca^{2+} influx, thus maintaining the Ca^{2+} gradient when the external Ca^{2+} concentration is low.

changes and shows the need for carefully timed observations.

From the manipulations described above, it seems likely that the establishment and maintenance of the Ca^{2+} gradient requires an influx of Ca^{2+} through Ca^{2+}-permeable channels. However, as pointed out by Harold (53), the importance of a Ca^{2+} influx through the apical membrane is called into question by the fact that hyphae can grow for considerable periods in the absence of exogenous Ca^{2+} (70, 137). In light of the above data, which suggest that localized influx is important, it is worth seeking an explanation consistent with observed growth in EGTA-containing media, which should ensure very low levels of exogenous Ca^{2+} and thus eliminate influx. During growth, the continually synthesized cell wall creates a dynamic barrier between the plasmalemma (the presumed site of Ca^{2+} influx) and the medium. Although this barrier is thin and of undefined permeability, its presence and continual secretion would generate a gradient of, e.g., EGTA between the medium and the plasmalemma. Depending on the dynamics of the apical wall, it is possible, but certainly not proven, that the EGTA is effectively absent from the vicinity of the plasmalemma. A supply of Ca^{2+} delivered from endogenous stores to the apical periplasmic space may thus be available for influx until the endogenous reserves are depleted. A valid model for a delivery system is the vesicles which secrete the wall material itself. Their lumen is developmentally continuous (via the Golgi apparatus) with that of the endoplasmic reticulum, which is likely to be a Ca^{2+}-sequestering part of the cell (see the section on maintenance of low cytosolic Ca^{2+} levels in fungi, above). Consequently, they provide a plausible carrier system from a probably Ca^{2+}-rich source to a localized sink where they could maintain a pool of Ca^{2+} inside the wall, away from exogenous EGTA, and available for subsequent influx through the plasmalemma (Fig. 5). This concept of Ca^{2+} "bootstrapping" is compatible with the general lifestyle of fungi. As a group they have evolved the ability to reutilize previously incorporated molecules from subapical regions to supply the growing tip and thus permit hyphal extension, and environmental exploration, under starvation conditions. If the wall vesicles do transport Ca^{2+}, their lack of staining with CTC could be explained by invoking a pH effect (see the section on membrane-associated Ca^{2+}, above). Whether such a mechanism can maintain a sufficiently high level of Ca^{2+} in a sufficiently large but localized region of the tip to be effective in replacing the normal gradient generated by Ca^{2+} influx cannot yet be determined because of technical limitations. This is not a trivial problem, since the essential portion of the gradient may only be a thin zone adjacent to the extending portion of the plasmalemma.

The Ca^{2+} gradient appears to enhance hyphal extension, ensure normal tapered hyphal morphology, and inhibit hyphal branching. Despite the above data, the complexity of hyphal growth ensures that we are only just beginning to gain an insight into the possible regulatory roles that Ca^{2+} gradients may play in the process.

Ca^{2+} Sequestered by Organelles

We have seen that hyphae contain substantial numbers of Ca^{2+} ions, probably sequestered into vacuoles, the endoplasmic reticulum, and mitochondria. These stores are likely to be important in setting up and maintaining the Ca^{2+} gradient of the tip and are presumably in some equilibrium with the free cytoplasmic Ca^{2+}. The key question is whether there is any special regulatory system which controls the release of these Ca^{2+} in a manner directly essential to tip growth. The most likely candidate for such a system, on the basis of our current understanding of intracellular Ca^{2+} regulation, is the IP_3 system.

IP_3-induced release of Ca^{2+} may be involved in hyphal growth. Lithium prevents complete dephosphorylation of IP_3 and thus blocks the phosphoinositide cycle, preventing IP_3-stimulated Ca^{2+} release (3). In fungi, lithium decreases the rate of tip extension and increases branching (52), implying that IP_3-mediated Ca^{2+} release, possibly from the vacuole (28), may be important in the regulation of hyphal growth. Similarly, Brunton and Gadd (14) showed that various components of the IP_3 cycle are important in yeast-hypha dimorphism in *Ophiostoma* species. However, it is not at all clear whether these effects are exerted directly on Ca^{2+} release from sequestration and tip growth or are more indirect. For example, the breakdown of phosphatidylinositol into IP_3 and diacylglycerol has been shown to be crucial for *Saccharomyces* cell division and thus may also be important in the mitotic cycle of other fungi (162). In a growing hyphal tip of a cenocyte, a proportion of the nuclei are mitotic. The number of nuclei in the tip remains constant, while some nuclei are left behind to populate subapical regions. Therefore, mitosis is associated with hyphal growth, and reduced growth in the presence of lithium may therefore be due to blocking of mitosis rather than to preventing the release of vacuolar Ca^{2+}. Thus, IP_3 appears to be involved in growth but may play only an indirect role in maintaining the Ca^{2+} gradient.

Ca^{2+}-Binding Proteins

To understand the function of Ca^{2+} in growth, Ca^{2+}-binding proteins must be identified and their functions must be determined. Calmodulin is thought to be a ubiquitous intracellular Ca^{2+}-binding protein which functions to mediate many Ca^{2+}-regulated processes in cells (23). The Ca^{2+}-calmodulin complex can either directly bind to a target protein and thus alter its activity, indirectly stimulate the target protein and thus alter its activity, or indirectly stimulate the target protein through a Ca^{2+}-calmodulin-dependent protein kinase. Calmodulin is present in a variety of fungi

(48, 114, 151), and there is evidence for protein regulation by phosphorylation in fungi (103, 147, 164). Inhibitors of calmodulin have been noted to increase the frequency of branching of *Fusarium* and *Neurospora* hyphae (114, 132), although branching of *Achlya* hyphae was not affected (54). In addition, tip extension was slowed by these inhibitors (114, 132). It is therefore possible that Ca^{2+}-influenced branching and tip extension are mediated via Ca^{2+}-calmodulin. However, this conclusion may be premature, since there are nonspecific effects of calmodulin antagonists (29, 91), including the induction of increased cytosolic free Ca^{2+} concentration (41). Furthermore, other Ca^{2+}-binding proteins which are likely to be involved in tip growth have been identified. For example, the *CDC24* gene encodes a possible Ca^{2+}-binding protein which is necessary for normal budding in *Saccharomyces* cells. Although the function of this protein is unknown, it is likely to bind, and therefore may be regulated by, Ca^{2+} (94). A similar protein may be required for tip extension or branching of hyphae. Ca^{2+} is also known to activate other proteins, although they may not directly bind Ca^{2+}. K^+ channels in the *Saprolegnia* plasma membrane are activated by Ca^{2+} (38). Blocking these channels with tetraethylammonium ions only transiently inhibits tip extension; therefore, these channels are not obligatory for growth. Ca^{2+} ions also stimulate the *Neurospora* plasma membrane proton-ATPase (92). This may function to stimulate efflux of Ca^{2+} ions from the cytoplasm, since proton gradients can drive Ca^{2+}/H^+ exchange. Thus, Ca^{2+} ions could regulate their own concentration.

Ca^{2+} Regulation of the F-Actin Network

F-actin is concentrated in growing fungal tips (56, 63, 136, 138), the extending ends of fission yeasts (17, 96), and the buds of budding yeasts (1). It is also concentrated in the apices of nonfungal tip-growing organisms (145). Actin is thought to play a multifunctional role in tip growth by coordinating tip morphogenesis, cell wall synthesis, cytoplasmic migration, and organelle positioning (58). The abundance of both actin and Ca^{2+} in growing hyphal tips, along with well-described direct and indirect Ca^{2+} regulation of actin networks (see below), makes Ca^{2+} control of tip growth via actin seem likely.

Ca^{2+} ions are known to regulate actin in a number of ways. They can bind directly to actin and affect monomer synthesis and conformation (7, 100). They can regulate the structure and hence the function of the actin network through a variety of actin-binding proteins which are controlled by Ca^{2+} (25, 163). Elevated levels of Ca^{2+} can also stimulate protein phosphorylation (i.e., via calmodulin), which may affect the actin network by phosphorylation of actin-binding proteins (11, 60, 111, 163) or by direct phosphorylation of actin, which may affect interactions of actin with actin-binding protein (120). Thus, the behavior of the apical actin network will depend on a balance of cooperative and competitive interactions among a variety of actin-binding proteins (34, 55, 69), many of which will be regulated by Ca^{2+}.

Ca^{2+} may regulate hyphal morphogenesis through control of the apical actin network. The absence of exogenous Ca^{2+} leads to depleted levels of membrane-associated Ca^{2+} (70, 171), the swelling of hyphal tips, and the loss or reduction of the apical cap of F-actin (70). Damage to F-actin, induced by cytochalasins, also results in bulbous tips (4, 46, 71, 160), implying that it is the loss of the actin cap which results in apical swelling. However, actin cap damage, induced by UV microirradiation and accompanied by an increased cytoplasmic Ca^{2+} concentration, results in cessation of growth but does not cause apical swelling (75). Under these circumstances, cell wall synthesis is likely to have stopped, since the apical concentration of wall vesicles is lost in response to A23187 elevation of the Ca^{2+} concentration (135). Apical swelling probably requires continued cell wall synthesis, as is demonstrated by the increase in calcofluor staining of swelling tips (71). Therefore, it is possible that, under low-Ca^{2+} conditions, loss of the actin cap is accompanied by continued wall synthesis, with the deformable wall becoming spherical as it lacks an actin cap for support. Conversely, actin cap damage, concurrent with an increase in the Ca^{2+} concentration, may result in the cessation of wall synthesis and hardening of the apical wall such that these tips would not swell. This may explain why two different results are observed when the Ca^{2+} gradient is disrupted.

During growth, the cytoplasm is continually moving forward with respect to the lateral cell wall and plasma membrane, in order to maintain its position in the tip (99). Ca^{2+} ions may be involved in regulating this migration, since Ca^{2+}-dependent polarized tipward cytoplasmic contractions were induced in *Basidiobolus* (98) and *Saprolegnia* (73) hyphae by UV microirradiation. The Ca^{2+} ionophore A23187 could induce similar contractions (82, 98). These contractions are likely to be actin-myosin based, since F-actin is concentrated in the contracted cytoplasm (73, 99). Furthermore, cytoplasmic motility in other systems, such as contraction in amoebae (153) and streaming in algae and higher plants (62, 89, 168), is also actin-myosin based. The high concentration of Ca^{2+}, seen in growing hyphal tips, may be regulating interactions between actin and myosin, possibly via phosphorylation-dephosphorylation (156), resulting in movement of cytoplasm.

CONCLUSIONS

We have summarized evidence which suggests that a tip-high gradient of cytoplasmic Ca^{2+} plays a regulatory role in hyphal tip growth in diverse fungi. We have also identified plausible ways in which these ions may themselves be regulated and may, in turn, modulate the tip growth process. While providing a critical evaluation of the data, we have adopted an advocacy approach to their interpretation. In doing this, we are mindful of ambiguities in the interpretation of most of the results under discussion. Some of these ambiguities are intrinsic to the tip growth process itself. It is very localized and dynamic and is undeniably influenced by many factors in the subtending regions of the hypha. Therefore, the process is difficult to investigate at sufficiently high resolution without disruption, and it is hard to separate direct from indirect effects. It is also likely that with a process as vital to the organism (indeed, to the entire fungal kingdom) as tip growth, evolution will have produced multiple backup systems such that perturbation of one component can be compensated for by other components. Such a concept has, in a commentary on cell locomotion, been likened to parallel processing in computational systems (10). This concept is particularly interesting because it means that collapse of the system (e.g., inhibition of tip growth) may not follow from disruption of one component which is normally involved. Compensation is easily envisaged in a multicomponent system, such as tip growth, because we know that the final result (the hypha) must be the result of a fine balance among numerous synthetic, pressure-regulatory, and extensibility-regulatory processes. Consequently, ex-

ceptional observations (e.g., the demonstration that a specific component is not essential in a specific organism) may not be proof of the normal rule.

Bearing in mind the above point, it is possible to identify numerous aspects of the Ca^{2+} story for which further work may be most rewarding. More higher-resolution studies of Ca^{2+} distributions in a greater range of demonstrably growing tips are essential. These studies must include the effects of modulating exogenous Ca^{2+} levels, and these experiments, in turn, must pay careful attention to the effects of other ions in the system, especially since the commonly used Ca^{2+} modulator EGTA is not specific for Ca^{2+} chelation (169, 170). These experiments are especially important because the limited data currently available suggest that comparable growth rates can occur in the presence of significantly different free cytoplasmic Ca^{2+} concentrations at the tip (see the section on calcium distribution, above) (Fig. 1). It is also important to conduct more detailed short-term analyses of perturbed tips, because they may differentiate between effects on the normal system and effects on an adapted system. These experiments may also help differentiate between direct effects on growing tips and abnormalities accumulating from general stress on the hyphae. Such studies should also focus on the initiation of tip growth, either during spore germination (e.g., the fucalean zygote system [84]) or during branch initiation. Further studies with assorted inhibitors may be rewarding but must include demonstration of the assumed effect of the inhibitors and a lack of effect on other possible targets (if possible), because few inhibitors are indeed specific for a single target. Additional studies, again on a greater diversity of species, by using patch clamp technology to identify and characterize the transport and regulatory characteristics and distributions of membrane channels are essential. These studies alone can identify the basis for likely localized ion fluxes. However, since at least some channels are stretch activated and others may not be revealed with the high-resistance seals that are usually used (93), a considerable diversity of measurement conditions must be used in these studies. If current indications of nonrandom distribution of at least some channels are found to be widespread, the mechanisms responsible for this distribution must be demonstrated. Another line of research which seems essential is the biochemical characterization of both the apical cytoskeletal apparatus and the apical cell wall and its enzymes. Such information is essential before realistic predictions of ion effects can be made.

At this stage in the ion regulation of tip growth, it seems premature to expect major advances from mutational analysis of the process. Devising screening procedures for tip growth-specific mutations, as opposed to general "sickness" in indirectly involved processes, seems very difficult. Disruption of specific targets is limited by both the very small range of currently known likely targets and the compensatory arguments made previously. Nevertheless, as we accumulate a longer list of likely structural, transport, and enzymatic molecules, the results of specific disruption experiments will be interesting if carried out with sufficient temporal and spatial resolution, as mentioned above.

We conclude that there is a substantial body of circumstantial evidence implicating Ca^{2+} ions in the regulation of the tip growth process of hyphae and other cell types. Details of their roles are largely lacking, and there may be alternative interpretations of some experimental data. Nevertheless, we believe that the weight of evidence necessitates the continued consideration of the impact of Ca^{2+} ions in the process and provides an adequate base to encourage further experimentation on the topic.

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